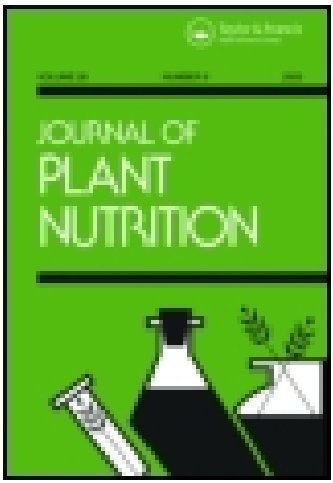

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ABSTRACT

We analyzed the effect of different copper (Cu) concentrations (0.10, 0.15, 0.20 and 0.35 mM) and time (1 day to 9 days) on several growth and biochemical parameters of roots and shoots of white lupin plants (*Lupinus albus* cv Estoril) grown in nutrient solution. A significant decrease in leaf fresh weight and leaf area was detected. Copper accumulated in the roots, and an impairment of nutrient translocation was only observed after six days at the highest Cu concentrations applied. A transient increase in the activity of polyphenoloxidase (EC 1.10.3.1) enforces a role for lignification as a defense strategy under enhanced Cu levels. The activities of several antioxidative enzymes were enhanced after Cu application. Our results indicate that *Lupinus albus* cv 'Estoril' is a rather resistant plant that can cope with moderate concentrations of copper, mostly by controlling up to a certain point, the uptake of excessive amounts of this metal.

Keywords: white lupin, copper, oxidative stress

INTRODUCTION

Copper (Cu) is an essential plant micronutrient that can become phytotoxic at elevated concentrations. Soils can be contaminated by copper and other heavy metals due to anthropogenic interferences, like industrial, mining and agricultural activities, namely the application of sewage sludges, organic residues,

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fertilizers, and fungicides. It is thus very important to study the interaction between heavy metals and plants, as it affects crop production and plant growth (Fageria, 2001).

Copper levels in soils are very variable, depending on soil type and characteristics (like acidity, organic matter content, texture, cationic exchange capacity and redox potential), but there is usually enough copper for an adequate nutrition of plants, at average concentrations between 1 and 50 $\mu g.g^{-1}$ (Thornton, 1979). The fraction bioavailable to plants is usually much lower and represents only a few percentages, at most, of total copper in the soil (Alva et al., 2004). Although copper can exist both in free ionic form (Cu^{2+}) and complexed in organic or inorganic forms, the organic complexes are much more abundant, as copper has a very high affinity for organic matter compared to other heavy metals (Adriano et al., 2004) with up to 98% of copper in soil solution being complexed with low molecular weight organic compounds (Fox and Guerinot, 1998).

In general, for healthy plants, copper can be present at concentrations between 5 and 20 $\mu g\ g^{-1}$ (on a dry matter basis), with symptoms of toxicity appearing above these values (Reuter and Robinson, 1997). It is reported to have a restricted mobility inside the plant, accumulating heavily in roots and to a much lesser extent in shoots. Furthermore, between 35 and 90% of copper in the leaves is located in the chloroplasts (Fernandes and Henriques, 1991).

Copper is an essential element in several plant metabolic processes as it is a prosthetic group of several enzymes and participates in photosynthesis and in lignification processes (Maksymiec, 1997). However, when plants are exposed to elevated copper concentrations, several metabolic processes are affected more or less seriously depending on the growth stage of the plant at the moment of copper application, on the duration of its effect and on the efficiency of the plants' defense strategies.

Usual visible symptoms of heavy metal toxicity include leaf chlorosis and inhibition of growth affecting both shoots and roots (Shaw, et al., 2004). Physiological activities affected by copper are photosynthesis, chlorophyll synthesis, respiration, carbon dioxide (CO_2) fixation, gas exchanges and other nutrient transport inside the plants (Fernandes and Henriques, 1991; Maksymiec, 1997). At the cellular level, excess copper can induce oxidative stress due to the production of reactive oxygen species (ROS) (Apel and Hirt, 2004), which, in turn, will affect proteins and cellular membrane lipids causing membrane damage, increasing its permeability, and lipid peroxidation (Vangronsveld and Clijsters, 1994). This effect is more apparent in roots as they are in direct contact with the metal.

In the presence of elevated concentrations of copper (as well as other metals), plants enhance several defence strategies. These can be of two types: avoidance, where plants avoid the uptake of excessive amounts of heavy metal and tolerance, where plants develop means to deal with the excessive uptake of the heavy metal (Vangronsveld and Clijsters, 1994; Clemens, 2001; Hall, 2002).

Although plants can use ligands, such as organic acids, metallothioneins, and phytochelatins to chelate heavy metals and eventually compartmentalize the ligand-metal complex, this defence mechanism is reportedly not very efficient for copper (Cobbett and Goldsbrough, 2002), and, for example, it has not been detected in *Arabidopsis thaliana* when it was grown under toxic copper concentrations (Wojcik and Tukiendorf, 2003).

The increase of ROS levels activates specific defense mechanisms, like the induction of antioxidative enzymes (Clijsters et al., 1999) the most important of which are superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), and guaiacol peroxidase (GPOD, EC 1.11.1.7) (Van Assche and Clijsters, 1990; Weckx and Clijsters, 1996).

The plant species used in the present work is *Lupinus albus* L. (white lupin). Nowadays, plants of the *Lupinus* genus are cultivated because of its large utilization as an animal feed, as green manure contributing to improved soil structure (Erbas et al., 2005) but also for human nutrition. As lupins are a leguminous that can use atmospheric nitrogen and are well adapted to adverse climate and soil conditions and to different landfill conditions such as excess nitrate and lime, high salinity and heavy metal concentrations, this species is an excellent candidate to increase the fertility of contaminated soils (Castaldi et al., 2005). Clemens et al. (2002) refers to *Lupinus albus* L. as an example of plants that use specific mechanisms to control root exudate production of organic acids that may help in increasing plant tolerance to high metal content. Zornoza et al. (2002) have reported that white lupin plants are relatively tolerant to cadmium-induced stress.

As white lupins appear to have the potential to improve heavy-metal contaminated soils, albeit not being a hyperaccumulator, and as plant responses to heavy metal stresses are very variable, we intended to clarify some of the defense mechanisms *Lupinus albus* uses against toxic copper concentrations. In this work, the effect of copper on white lupin plants (*Lupinus albus* L. cv 'Estoril') in hydroponic culture was studied.

MATERIAL AND METHODS

Plant Material

White lupin seeds (*Lupinus albus* L. cv 'Estoril') were germinated in filter paper soaked in deionized water for ten days. Germinated seeds were transplanted to plastic boxes with perforated covers, containing 2.5 L of aerated Hoagland nutrient solution, under controlled conditions (temperature between 18 and 25°C, 14 hour photoperiod, and 55% relative humidity). The nutrient solution was renewed weekly. Five weeks after transplantation, the following copper concentrations (as $CuSO_4$) were added to the nutrient solutions: 0.1 mM,

0.15 mM, 0.2 mM, and 0.35 mM. The control boxes contained the original 0.1 μM copper concentration. Each copper treatment was performed in triplicate for a total of 36 plants per treatment.

Experimental Determinations

All analytical determinations were performed in triplicate three, six, and eight days after copper application, except the enzymatic determinations that were performed after one, two, three, four, seven, and nine days. For leaf area determination, all the leaves from four plants in each treatment were harvested, followed by scanning and measuring the dark area using computer software (Delta-T scan 2.03; Delta-T Devices LTD, Cambridge, UK), giving results in arbitrary units of area. Main root length was measured in all plants, using a ruler. Chlorophyll content was determined using the portable SPAD-502 device (Konica Minolta Corporation, Tokyo, Japan) giving the measurements in SPAD units that are well correlated to chlorophyll content. A previous calibration was performed where chlorophyll concentration measured according to Abadia and Abadia (1993), was plotted against SPAD measurements. A straight line was fitted to the results ($y = 0.8151x + 20.373$) with an $r^2 = 0.98$.

Dry weight was recorded after the plants were harvested and dried at 100°C to a constant weight. For the determination of calcium (Ca), Cu, iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), and zinc (Zn) concentrations in roots and leaves, samples of dried plant material were ashed at 480°C in a muffle furnace, digested twice in 10 mL of 3 M hydrochloric acid (HCl) at 90°C and analyzed by flame atomic absorption spectrophotometer (Unicam Solaar M, Thermo Fisher Scientific, Waltham, MA, USA). Phosphorus was determined by the molybdovanadate colorimetric method (using a Hitachi U-2000 UV/Vis Spectrophotometer, Hitachi Ltd., Tokyo, Japan).

Enzyme Analysis

Crude extracts were obtained by maceration of ca. 0.5 g of plant material with 2% (v/v) insoluble polyvinylpolypyrollidone and 5 mL of extraction buffer [100 mM Tris-HCl buffer, pH 7.5, containing 3 mM dithiothreithol and 1 mM ethylenediaminetetraacetic acid (EDTA)]. The extract was centrifuged at 10000 $\times$ g for 30 minutes, at 8°C (Sigma 3–18K, Osterode am Harz, Germany) and the supernatant was then filtered through 0.20 μm filters. Guaiacol peroxidase (EC 1.11.1.7) activity was measured according to the modified method of Adams (1978). The increase in the absorbance at 420 nm was measured for 2 minutes in a reaction medium containing 30 mM guaiacol and 4 mM of hydrogen peroxide (H_2O_2), in a 0.2 mM sodium acetate buffer (pH 6.0). Enzymatic activity is defined as the consumption of 1 μmol of guaiacol per minute per ml at room

temperature, under the assay conditions, using for tetraguaiacol the value of $\varepsilon = 26.6\ mM^{-1} \cdot cm^{-1}$. Polyphenol oxidase (PPO, EC 1.10.3.1) activity was measured according to the modified method of Oktay et al. (1995), by measuring the increase in absorbance at 420 nm for 2 minutes, using a reaction solution containing 30 mM catechol in 50 mM phosphate buffer (pH 7.0). Enzymatic activity is defined as the catechol absorbance variation per minute per ml at room temperature, under the assay conditions. Catalase (EC 1.11.1.6) activity was determined using a modified Aebi (1983) method, measuring the decrease in absorbance at 240 nm for 2 minutes, in a solution containing 10 mM of H_2O_2 in 50 mM phosphate buffer (pH 7.0). CAT enzymatic activity is defined as the consumption of 1 μmol of H_2O_2 per minute per ml at room temperature, under the assay conditions, using a ε for H_2O_2 of 39.4 $mM^{-1} \cdot cm^{-1}$. Superoxide dismutase (EC 1.15.1.1) activity was determined according to the modified Rubio et al. (2002) method, measuring the increase in absorbance at 550 nm for 2 minutes in a solution containing 0.5 mM xanthine, 0.05 mM ferricytochrome-C, 0.1 mM EDTA and xanthine-oxidase in 100 mM potassium phosphate buffer (pH 7.6). Enzymatic activity is defined as the quantity necessary for the inhibition of 50% of ferricytochrome-C reduction per minute under the assay conditions. All the enzyme activity results are expressed in relative activity, A/A_0, where A is the measured enzyme activity as defined above and A_0 is the enzyme activity for the control, at the beginning of the experiment.

Statistical Analysis

The statistical analysis was performed using the SPSS 10.0 software (SPSS Inc, Chicago, IL, USA). The results were subjected to a one-way ANOVA, using the Tukey test to check significant differences between means ($P < 0.05$).

RESULTS

Copper contents in both leaves and roots significantly increased with copper concentration in the nutrient solution and with time (Figure 1A and B). Under control conditions and at 0.10 mM application, the values of Cu content in roots and leaves were in the same range. After six days at 0.10 mM, the values for shoots and roots were 45 mg kg^{-1} DW and 51 mg kg^{-1} DW, respectively. A supply of 150 μM Cu and more resulted in a decreasing root-shoot transfer, e.g., after eight days at a copper concentration of 0.20 mM, a maximum of 123 mg kg^{-1} DW in shoots and 4094 mg kg^{-1} DW in roots was measured, compared to 40 mg kg^{-1} DW and 42 mg kg^{-1} DW, respectively, in the control.

In Tables 1 and 2 the results for the mineral content of leaves and roots are presented, for those elements where significant changes were detected. In

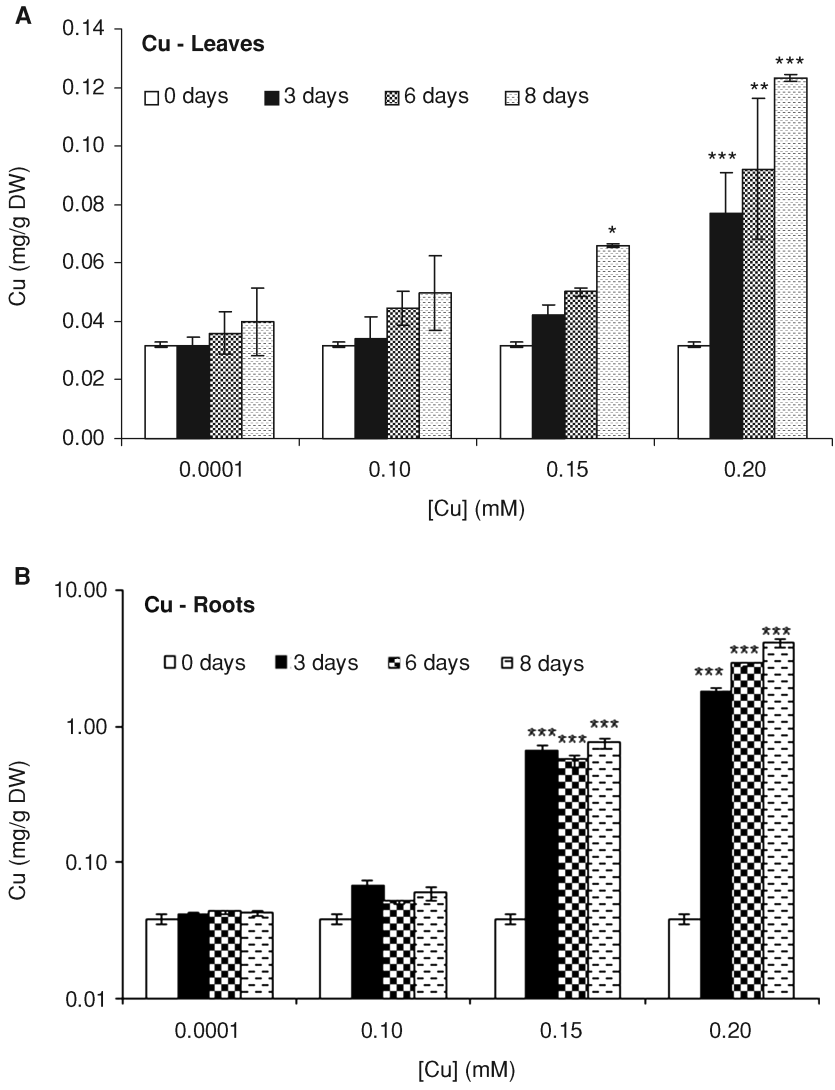

Figure 1. Copper content as a function of time and copper concentration in nutrient solution for (A) leaves and (B) roots. Significant differences in relation to the control (0.0001 mM): ***P < 0.001, **P < 0.01, *P < 0.05.

both plant organs K, Mg, and P contents were not affected by the Cu treatment. In roots the major difference was found in the Fe content, which increased under Cu exposure. In leaves, a decrease in Fe and Mn content together with an increase in Zn content were noticed after Cu application.

Table 1
Element concentration in white lupin leaves (Ca, Fe, Mn, Na, Zn)

		Element concentration (mg.g^{-1} DW)			
Element	Days	[Cu] = 0.0001 mM	[Cu] = 0.1 mM	[Cu] = 0.15 mM	[Cu] = 0.2 mM
Ca	0	9.63 ± 0.65	9.63 ± 0.65	9.63 ± 0.65	9.63 ± 0.65
	3	9.51 ± 1.18	10.02 ± 0.65	$11.52 \pm 0.17^{*}$	$11.58 \pm 0.39^{*}$
	6	10.16 ± 1.02	10.20 ± 0.27	11.76 ± 0.24	12.06 ± 1.06
	8	10.28 ± 1.17	10.17 ± 1.36	11.11 ± 0.59	12.53 ± 0.22
Fe	0	0.202 ± 0.011	0.202 ± 0.011	0.202 ± 0.011	0.202 ± 0.011
	3	0.250 ± 0.004	0.217 ± 0.059	0.206 ± 0.051	0.189 ± 0.027
	6	0.275 ± 0.009	0.240 ± 0.004	0.231 ± 0.004	0.209 ± 0.066
	8	0.283 ± 0.008	0.268 ± 0.008	$0.245 \pm 0.018^{*}$	$0.216 \pm 0.016^{**}$
Mn	0	1.542 ± 0.094	1.542 ± 0.094	1.542 ± 0.094	1.542 ± 0.094
	3	1.780 ± 0.041	1.579 ± 0.051	1.536 ± 0.076	1.467 ± 0.250
	6	1.860 ± 0.013	$1.563 \pm 0.139^{*}$	$1.405 \pm 0.023^{**}$	$1.305 \pm 0.094^{***}$
	8	2.060 ± 0.056	$1.596 \pm 0.154^{*}$	$1.359 \pm 0.204^{**}$	$1.347 \pm 0.065^{**}$
Na	0	0.205 ± 0.058	0.205 ± 0.058	0.205 ± 0.058	0.205 ± 0.058
	3	0.223 ± 0.016	0.199 ± 0.065	0.197 ± 0.052	0.201 ± 0.032
	6	0.261 ± 0.036	0.212 ± 0.009	0.206 ± 0.001	$0.194 \pm 0.027^{*}$
	8	0.286 ± 0.013	0.229 ± 0.004	0.219 ± 0.076	0.200 ± 0.007
Zn	0	0.132 ± 0.002	0.132 ± 0.002	0.132 ± 0.002	0.132 ± 0.002
	3	0.125 ± 0.004	0.133 ± 0.014	0.143 ± 0.007	0.145 ± 0.001
	6	0.126 ± 0.013	0.141 ± 0.011	0.148 ± 0.016	0.157 ± 0.008
	8	0.123 ± 0.015	0.132 ± 0.003	$0.143 \pm 0.001^{**}$	$0.175 \pm 0.001^{***}$

Significant differences in relation to the control (0.0001 mM): $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

The root length was measured at the 3, 6, and 8 day interval for all the studied copper concentrations but only after eight days the root length was significantly lower than the control (Figure 2). No significant differences were found for root fresh weight, nor for root dry weight both as a function of Cu and time (results not shown). At the lowest studied copper concentration of 0.10 mM lupin plants showed few visible toxicity symptoms, like slightly stunted growth. A measurable consequence of stunted growth is the decrease in shoot fresh weight. In Figure 3A we have a semi-logarithmic plot of leaf fresh weight against copper concentration in the nutrient solution. We fitted exponential correlations to the experimental data, obtaining good correlation coefficients, with increasingly negative slopes (given by $-k$ in the $y = \mathrm{e}^{(-kx)}$ model), which indicates that the decrease of leaf fresh weight is analogous to a first order reaction kinetics, and dependent on copper concentration. The dependence on time (after application of copper) can be shown by a plot of the obtained k values against time, yielding a straight line with an $r^2 = 0.992$

Table 2
Element concentration in white lupin roots (Ca, Fe, Na)

Element	Days	Element concentration (mg.g^{-1} DW) [Cu] = 0.0001 mM	[Cu] = 0.1 mM	[Cu] = 0.15 mM	[Cu] = 0.2 mM
Ca	0	3.35 ± 0.31	3.35 ± 0.31	3.35 ± 0.31	3.35 ± 0.31
	3	4.61 ± 0.24	3.38 ± 0.33**	3.44 ± 0.30**	3.93 ± 0.06*
	6	4.89 ± 0.05	4.51 ± 0.47	4.58 ± 0.50	3.97 ± 0.27
	8	4.69 ± 0.07	4.12 ± 0.09	4.54 ± 0.10	4.53 ± 0.16
Fe	0	3.014 ± 0.272	3.014 ± 0.272	3.014 ± 0.272	3.014 ± 0.272
	3	3.201 ± 0.026	4.061 ± 0.117**	4.014 ± 0.335**	4.355 ± 0.051***
	6	3.226 ± 0.233	4.164 ± 0.290*	5.016 ± 0.250***	5.483 ± 0.488***
	8	3.279 ± 0.293	4.391 ± 0.023**	5.880 ± 0.238***	7.245 ± 0.255***
Na	0	0.450 ± 0.015	0.450 ± 0.015	0.450 ± 0.015	0.450 ± 0.015
	3	0.411 ± 0.042	0.396 ± 0.037	0.418 ± 0.019	0.426 ± 0.011
	6	0.397 ± 0.006	0.397 ± 0.031	0.428 ± 0.021	0.438 ± 0.030
	8	0.328 ± 0.031	0.326 ± 0.009	0.359 ± 0.006	0.378 ± 0.020*

Significant differences in relation to the control (0.0001 mM): ***P < 0.001, **P < 0.01, *P < 0.05.

(Figure 3B). We applied the same statistical treatment to leaf dry weight results at 3, 6 and 8 days after copper application, and the results are shown in Figure 3A. The increase in the slope of the straight lines shows that there is an increase in dry weight both with copper concentration and time, as can be

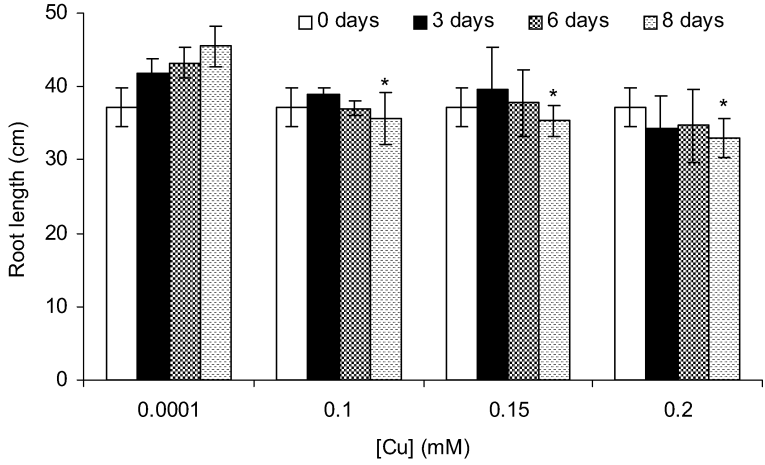

Figure 2. Root length as a function of time and copper concentration in nutrient solution. Significant differences in relation to the control (0.0001 mM): ***P < 0.001, **P < 0.01, *P < 0.05.

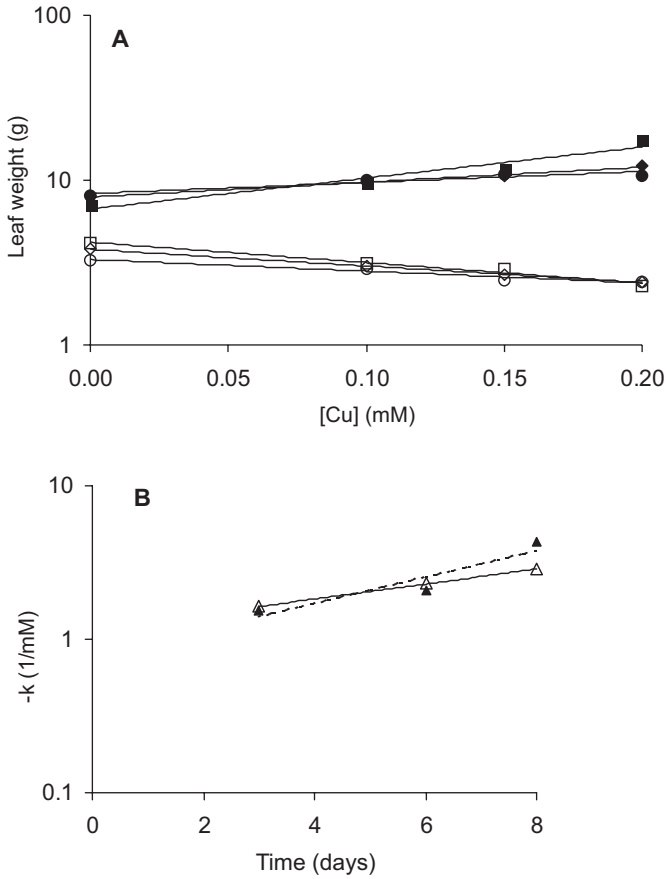

Figure 3. Leaf weight as a function of copper concentration. Fresh weight (open symbols): Plot A—Exponential correlations fitted to the experimental data: 3 days (○)–$y = 3.2997e^{-16256x}$, $R^2 = 0.934$; 6 days (◇)–$y = 3.7985e^{-23127x}$, $R^2 = 0.996$; 8 days (□)–$y = 4.2033e^{Z8493x}$, $R^2 = 0.976$. Plot B–k values from plot A against time (Δ): $y = 1.1642e^{0.1127x}$, $R^2 = 0.997$. Dry weight (solid symbols): Plot A—Exponential correlations fitted to the experimental data: 3 days (●)–$y = 8.2979e^{15423x}$, $R^2 = 0.874$; 6 days (♦)–$y = 7.8964e^{2082x}$, $R^2 = 0.997$; 8 days (■)–$y = 6.6824e^{43248x}$, $R^2 = 0.946$. Plot B–k values from plot A against time (▲): $y = 0.7834e^{0.1978x}$, $R^2 = 0.881$.

seen in Figure 3B. Regarding the leaf area, measurements presented in Figure 4 demonstrate that there is a significant decrease of this parameter both with copper concentration and with time.

Other visible symptoms like leaf chlorosis started to be noticed after the third day of copper application and were more pronounced for the highest copper concentrations. Nevertheless, no significant differences were found

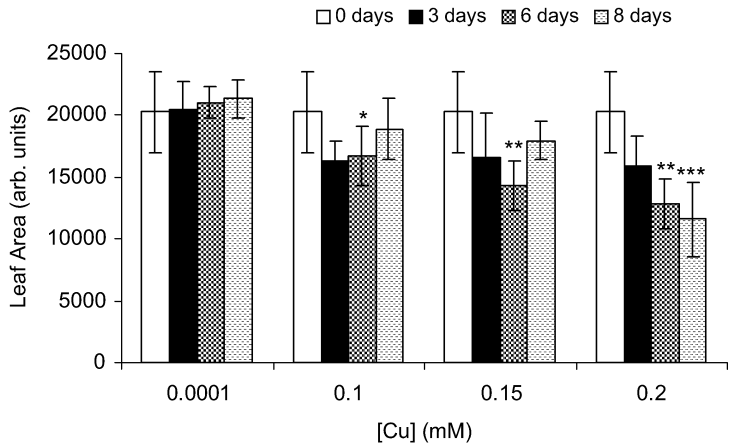

Figure 4. Leaf area (measured in arbitrary units) as a function of time and copper concentration in nutrient solution. Significant differences in relation to the control (0.0001 mM): $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

regarding to chlorophyll levels with the different copper treatments in the time period studied. There is however a noticeable negative correlation between chlorophyll levels (measured as SPAD units) and % of dry weight as shown in Figure 5.

The activities of the enzymes analyzed in this study in white lupin leaves, PPO, CAT, GPOD, and SOD are presented in Figure 6A to D. PPO activity increased transiently under Cu stress. For the other enzymes we can see that there was a strong increase in their activities, in white lupin leaves. An early rise in all activities was noticed, but this was transiently for CAT and SOD

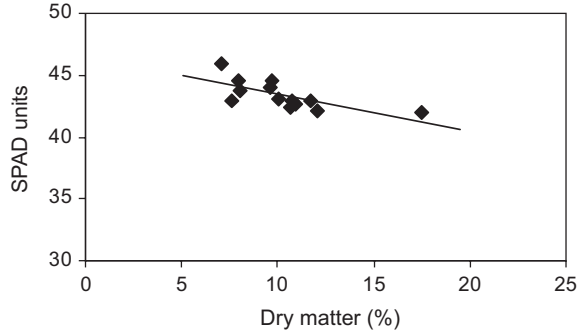

Figure 5. Correlation between chlorophyll concentration (measured as SPAD units) and leaf dry weight. $y = -0.302x + 46.455$, $R^2 = 0.505$.

(Figure 6B and D). GPOD activity increased immediately in a dose and time dependent manner (Figure 6C).

DISCUSSION

Excess copper, like excess in other heavy-metals, can cause harmful effects on plants, but the observable consequences of these effects vary according to plant species.

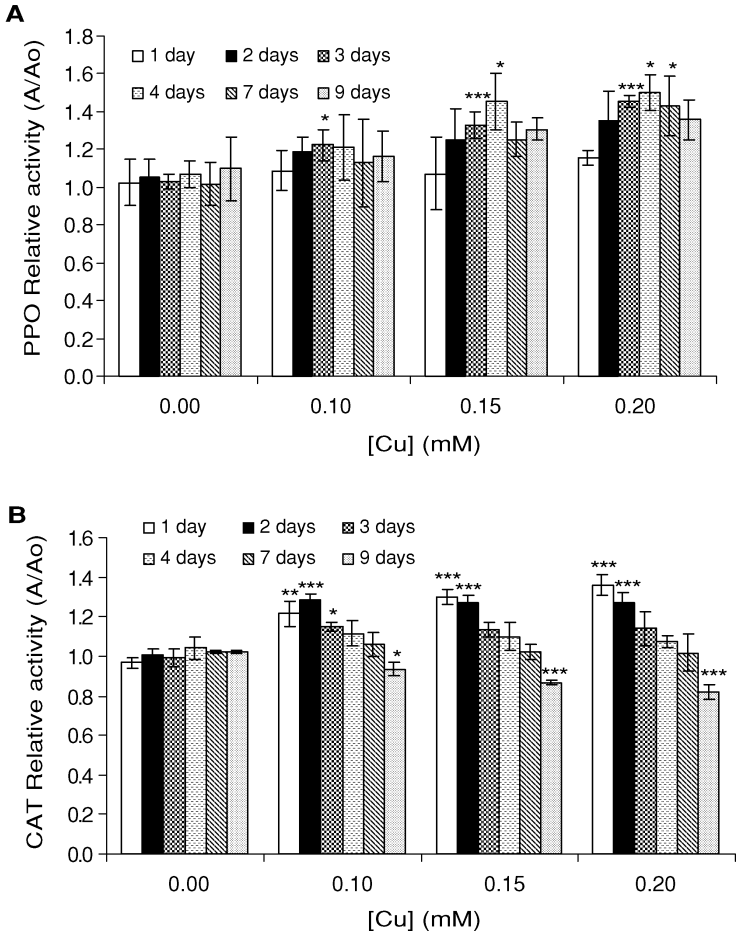

Figure 6. Enzyme relative activities in leaves as a function of time and copper concentration in nutrient solution. (A) polyphenoloxidase (PPO), (B) catalase (CAT), (C) guaiacol-peroxidase (GPOD), (D) superoxide-dismutase (SOD). Significant differences in relation to the control (0.0001 mM): $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. *(Continued)*

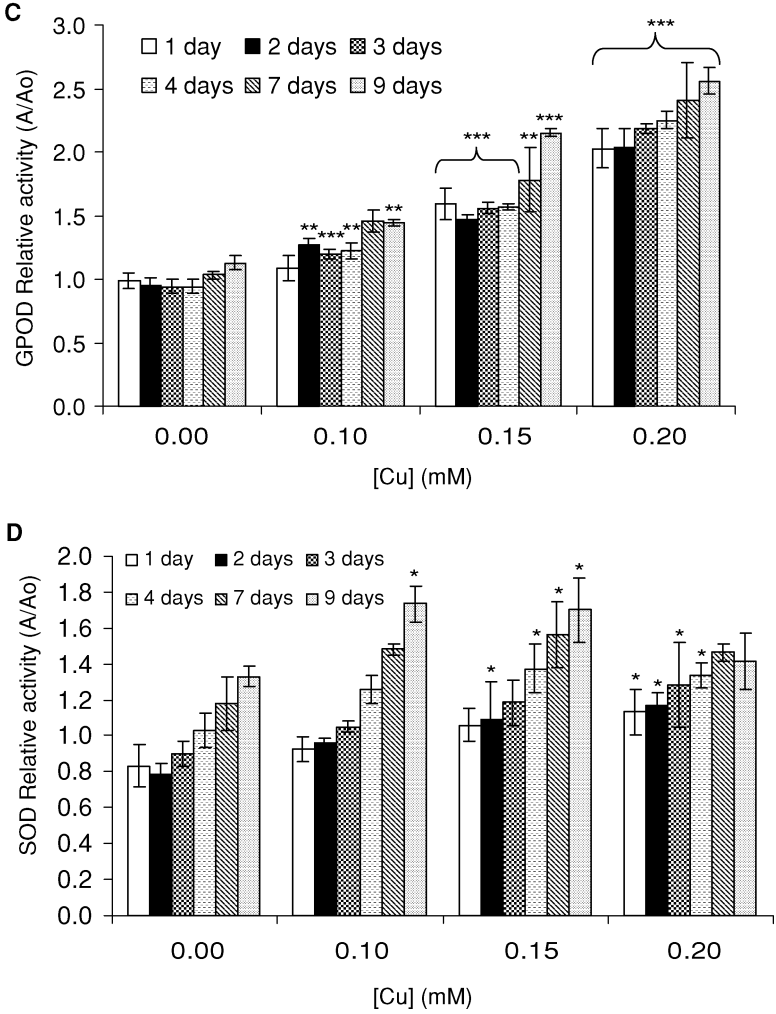

Figure 6. (Continued)

The white lupin plants used in this study accumulated much more copper in roots than in shoots, as expected. For the copper-tolerant *Elsholtzia haichowensis* values of around 50 mg kg^{-1} DW for shoots and slightly above 10000 mg kg^{-1} DW for roots, for the same six days and the same copper concentration (0.10 mM) in the nutrient solution were measured (Lou et al., 2004). In our study, no significant differences were found under these conditions as compared to the controls. It thus seems that *Lupinus albus* cv 'Estoril' has efficient mechanisms to exclude or avoid excess uptake of copper by the

roots. However, above a certain threshold value (0.15 mM Cu external concentration), this mechanism probably breaks down, and root copper content increases a lot, which is not immediately seen in shoots (Figure 1). So, the roots can act as a double barrier, first controlling the uptake of copper and then limiting its translocation to upper plant parts. Gonnelli et al. (2001) concluded that copper-tolerant populations of *Silene paradoxa* relied on an avoidance mechanism to counter excess copper and van Hoof et al. (2001) also reported that copper uptake was inversely proportional to copper tolerance, suggesting that copper tolerant species either avoided copper uptake or promoted copper efflux.

When plants are grown in phytotoxic concentrations of heavy metals, the roots are usually the first organs to be affected as they are in direct contact with the metal. Our results show that root length was only significantly lower than the control after eight days of copper application. Root length decrease is a frequently described consequence of copper toxicity, and has been reported in several plant species, like in rapeseed (Baryla et al., 2000), in *Silene paradoxa* (Gonnelli et al., 2001) and even in copper-tolerant ones like *Elsholtzia haichowensis* (Lou et al., 2004). However, although there was a slight decrease in root length there were no significant differences in both root fresh weight and root dry weight. This seems to indicate that a large percentage of copper was retained in the root cell walls without seriously affecting root metabolism. This is a very important and common strategy plants use to cope with heavy metal stress.

Shoot biomass showed a significant decrease with increasing copper concentrations while shoot dry weight significantly increased revealing a decrease in plant water content, which can, consequently, affect cellular turgor and cell enlargement. Leaf area also decreased, but the differences were only significant after six and eight days at an exposure to 0.2 and 0.35 mM Cu. This coincides with a strong increase of the Cu content in the leaves, indicating that limitation of Cu translocation by the roots is exceeded.

Although a decrease in chlorophyll content in leaves is a commonly described effect of excess copper action (Chatterjee et al., 2006), either due to direct metal influence on the formation of chlorophyll or due to the decrease in iron uptake, we observed no significant differences in chlorophyll content. Nevertheless there was a significant decrease in iron content in shoots and a significant increase in iron content in roots. This indicates that, even if iron uptake by the roots was not affected, its translocation to upper plant parts was disturbed, but even at the maximum copper concentration of 0.20 mM and after eight days of treatment the iron content in shoots was still above critical levels (Reuter and Robinson, 1997).

It has been reported that the competitive interaction between copper and calcium and/or magnesium could induce stunted growth usually observed in

copper-stressed plants (Maksymiec, 1997), as copper can displace calcium ions. However, in our results, we did not detect any difference in Mg levels and only a slight decrease in calcium content in roots and a small increase in leaves. Mocquot et al. (1996) observed an increase in calcium and magnesium levels with increasing copper concentrations in young maize leaves, while Ouzounidou et al. (1998) found a decrease in Ca, Mg, Fe, K and Na in both roots and leaves of spinach grown in 0.160 mM copper. Yang et al. (2002) reported changes in nutrient uptake both in roots and in shoots of Cu-tolerant *Elsholtzia splendens*. These results indicate that the elemental profile in Cu stressed plants is dependent on the experimental set-up (like Cu concentration applied, exposure time, growth conditions), but also on the plant species and organ investigated.

As mentioned before, there was also a significant increase in iron content in the roots together with a slight decrease in the leaves. A similar effect was noticed for manganese with a significant decrease in leaf manganese content. The opposite effect was noticed for zinc with an increase in zinc levels in the leaves, but although there is an apparent decrease in root zinc concentration it is not statistically significant. Changes in translocation, only observed in our study after six days, might be caused by a perturbation of the controlled transport of elements. Probably at this exposure period, structural damage to the root membranes occurs, leading to an impairment of transport processes. Structural changes to membrane lipids have been observed under Cu stress (Padua et al., 2003).

It is well-known that excess copper can induce the formation of harmful ROS, leading to lipid peroxidation (Cuypers et al., 2000). It is clear that excess of ROS can be harmful for the plant cells, however recent findings suggest that H_2O_2 is an important signaling molecule in plant stress responses (Neill et al., 2002). This knowledge implies that the amount of ROS in the plant must be controlled. Besides functioning in signaling, H_2O_2 also acts as a substrate in stress related biosynthetic pathways like lignification (Mika et al., 2004). Lignification has been postulated to be an important detoxification process in plants under Cu stress (Cuypers et al., 2002). PPO is an enzyme involved in lignification processes. The activity of PPO in white lupin leaves was measured and we observed a transient increase in this enzyme activity. Gonnelli et al. (2001) also reported an increase in this enzyme activity and associated it with cell wall stiffening processes.

The ROS and peroxide scavenging enzymes are induced when plants are grown under toxic copper concentrations (Van Assche and Clijsters, 1990; Cuypers et al., 2000; Gupta et al., 1999). This indicates that plants possess an antioxidative defense system to control the cellular levels of ROS. There have been, however, contradictory reports as sometimes the activities of these enzymes decrease with the increase in heavy metal concentration but this can be caused by either the direct action of ROS on the proteins or on the inhibition

of protein synthesis (Mazhoudi et al., 1997). Our results for CAT activity seem to show both these two effects. The activities measured after one, two, and three days increase for all the copper concentrations, probably to eliminate the excess hydrogen peroxide induced by copper, but at longer exposition times the activities return to control levels or even lower, as in the case of the 9th day measurements. This can be caused by the negative effect of copper-induced ROS on the enzyme proteins. The same pattern of early activation of CAT followed by a decrease in activity has been observed before in bean leaves (Weckx and Clijsters, 1996). Other authors have reported different responses of catalase to copper excess. Mazhoudi et al. (1997) detected no differences in tomato leaves after seven days growing in 50 μM copper while Chen and Kao (1999) reported a decrease in the activity after 48 hours in detached rice leaves. The duration of the heavy metal action can thus be a very important factor in the activation/inhibition processes in enzymes. Enhancement of CAT activity clearly is an early response to Cu application in our experimental set-up, but it is sensitive to prolonged Cu exposure (Figure 6B). GPOD activity also increased immediately with copper concentration. Moreover, it increased constantly with time, showing that this enzyme is more resistant to enhanced Cu levels. Clearly, together with catalase, GPOD seems to be important in controlling the excess of H_2O_2 that copper can induce. The observed results indicate that there is a strong dose-response relationship regarding GPOD activity in the leaves and copper concentration in the nutrient solution, and this behaviour has also been reported in maize plants (Mocquot et al., 1996) and in bean plants (Cuypers et al., 2002). When grown in excess copper concentrations, Mazhoudi et al. (1997) reported no changes in the activity of peroxidase in tomato leaves but an increase in this enzyme activity is more common and has been reported, for example, by Gonnelli et al. (2001) in certain populations of *Silene paradoxa* and by Mocquot et al. (1996) in maize leaves. We also did an electrophoretic analysis of isoperoxidase patterns (results not shown) but observed no difference in the patterns for the different copper concentrations suggesting that, although peroxidase activity increased it was not due to the appearance of new isoforms. New isoperoxidase forms have been previously reported in *Phaseolus vulgaris* grown under a copper concentration of 15 μM (Cuypers et al., 2000). On one hand, our results for SOD activity show that there is also an early increase in its activity, but only at higher Cu exposure (Figure 6D). On the other hand, the activity tends to decrease again at longer exposure times at the highest external copper concentration. SOD obviously plays a role in copper induced detoxification in white lupin plants. Several authors have reported no changes in SOD activity under copper stress in *Phaseolus vulgaris* (Weckx and Clijsters, 1996) and in detached rice leaves (Chen and Kao, 1999) while others report an increase in SOD activity, like in *Silene paradoxa* (Gonnelli et al., 2001) and *Elsholtzia splendens* (Peng et al., 2006).

CONCLUSIONS

The results presented in this work seem to show that *Lupinus albus* cv 'Estoril' is a rather resistant plant that can cope well with moderate concentrations of copper in nutrient solution, mostly by controlling, up to a certain point, the uptake of excessive amounts of this metal. Furthermore, to control cellular damage induced by elevated Cu levels, white lupin plants have an efficient defense mechanism against oxidative stress. These cellular responses (activities of CAT, GPOD, SOD) are early responses that are affected before morphological effects like leaf area and copper content are observed. Our results show that white lupin possesses several strategies to cope with Cu stress, and thus may have the potential to improve heavy-metal contaminated soils, but further investigation is required to unravel the underlying mechanisms.

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